INSTITUUT VOOR PLANTENZIEKTENKUNDIG ONDERZOEK
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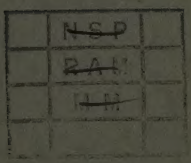
DIRECTEUR: Dr. J. G. TEN HOUTEN

MEDEDELING No 171

THE INOCULATION OF TOBACCO CALLUS TISSUE WITH TOBACCO MOSAIC VIRUS

DOOR

B. KASSANIS, T. W. TINSLEY AND FREDERIKA QUAK



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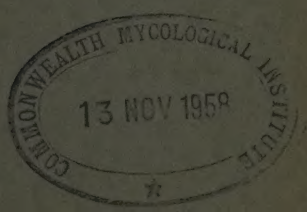

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THE INOCULATION OF TOBACCO CALLUS TISSUE WITH TOBACCO MOSAIC VIRUS

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(With Plate 2)

Although cultures of normal and conditioned tobacco callus tissue occasionally became infected when dilute solutions of tobacco mosaic virus was poured over them, injuries were usually required, and the number of infections depended on the type and number of injuries. Tissues infected by superficial injuries usually became virus-free after subculturing, whereas those infected by needle-prick remained infected permanently. Although no plasmodesmata were found joining cells in the tissue cultures, tobacco mosaic virus moved between them at a rate of about 1 mm. per week, approximately the same rate as it moves through cells of the leaf parenchyma.

Tumorous tissue from tobacco plants infected with tobacco mosaic virus was still infected after serial propagation for 8 years (Kassanis, 1957), but there is no information that plant-tissue cultures mechanically inoculated with viruses will also remain infected for long periods. Segretain (1943) infected some tobacco stems cultured in sterile media by pouring inoculum of tobacco mosaic virus over the stems, or by adding it to the media, but the callus tissue which was formed subsequently was not recultured and tested. Later Morel (1948) showed that tobacco tumorous tissue inoculated mechanically with tobacco mosaic virus contained the virus 4 months later, but his tests did not show whether the virus was restricted to the inoculated cells or had infected the whole culture.

The morphology of tissue cultures differs greatly from that of growing plants. Perhaps the most significant differences, as far as infection with viruses is concerned, are that the tissue cultures lack both a cuticle and an organized vascular system, which are important in affecting the entry of viruses into plants and their spread through plants. Because of these differences we have examined the mechanism of infecting tissue cultures and the spread of viruses in them. Although they are not tissue cultures in the true sense, we also included excised tomato root tips in our experiments, because White (1934), who found that they remained infective when excised from infected plants and propagated serially, failed to infect them mechanically.

MATERIALS AND METHODS

The tobacco tissue cultures, kindly supplied by Dr G. Morel, were his conditioned and normal callus (Morel, 1948). A culture with a very loose growth habit was selected from the conditioned callus (Pl. 2, fig. 1). This proved to be very useful for making suspensions of tissue cells in liquid media. Media of the composition described by Morel (1948) were used except that the conditioned tissue was grown in an agar medium with 2% glucose and 0·5 g./l. KH_2PO_4. The tissues were grown in the environmental conditions described by Kassanis (1957).

The normal callus tissue requires coco-nut milk in the medium for satisfactory growth. The milk is usually obtained from green coco-nuts, but as these were not available extracts were made from the kernels of mature ones. The white flesh was macerated in a blender, with 2 ml. of water per 1 g. of fresh material, the pulp pressed through muslin and the fluid left to stand for 2 hr. to allow the fat to separate. The bulk of the fatty layer was skimmed off and the fluid centrifuged to remove the remaining fat. The extract was brought to the boil and the coagulated proteins removed by centrifugation. The supernatant fluid was kept frozen until required.

The inoculum was sap from tobacco plants infected with the Rothamsted type strain of tobacco mosaic virus. The sap was mixed with an equal volume of phosphate buffer pH 7·0, centrifuged, and the supernatant fluid filtered through a sterile L3 filter candle. Infectivity tests on the filtrate showed that little virus was lost during filtration. All infectivity tests were made on plants of *Nicotiana glutinosa* L. The statistical analysis was done on transformed lesion numbers (Kleczkowski, 1949).

The tomato root culture was obtained from tomato seeds (*Lycopersicon esculentum* L.), var. Kondine Red which had been sterilized for 10 min. in 5% calcium hypochlorite and germinated in White's medium (White, 1943) made up in 0·8% agar. Tips of the roots were excised and thereafter cultured in liquid medium and maintained in an incubator at 24° C.

Inoculation of whole tissue cultures

Growing plants can be infected with viruses only through injured cells, but the observations of Segretain (1943) suggested that tissue cultures might be infected without abrasion. To investigate this, four different methods of inoculation were tried using conditioned tissues: (*A*) The inoculum was poured with care on to the surface of the tissues; (*B*) tissues were soaked for 10 min. in a Petri dish containing inoculum, rinsed in water and replaced on the medium; (*C*) tissues were pricked thirty times with a fine needle dipped in the inoculum; (*D*) tissues were rubbed with a micro-spatula dipped in inoculum containing Celite. The tissues were not inoculated within 10 days of subculturing, to allow to recover from wounds. In treatments *C* and *D*, as in *B*, the tissues were temporarily placed in Petri dishes.

Six weeks after the inoculation, the tissues were washed in running tap water and each piece macerated and tested separately for its infectivity on four half leaves of *Nicotiana glutinosa*.

The results (Table 1) show that the most infective extracts were from tissues given treatments *B*, *C* and *D*, and that the numbers of lesions and degree of injury to the tissue are correlated; treatments *C* and *D* gave more lesions than *B*. The few lesions produced by treatment *A* could result from residual inoculum remaining after the tissues were washed, but the possibility that some cells became infected cannot be excluded, especially as one of the replicates gave as many as forty-one lesions.

This experiment was repeated to find out how many of the inoculated tissues were permanently infected. Six weeks after inoculation each tissue was divided into four equal portions, three of which were recultured and the fourth tested for infectivity. The results of this test were very similar to those reported in Table 1. Some of the eighteen progenies taken from each treatment failed to grow and were discarded; treatments *C* and *D* had the most failures possibly because they caused most injury.

TABLE 1. *The infectivity of conditioned tissues 6 weeks after inoculation with tobacco mosaic virus*

Method of inoculation	Average number of lesions per half leaf of *N. glutinosa* produced by six replicated tissues					
	1	2	3	4	5	6
(*A*) 0·05 ml. of inoculum poured on uninjured tissues	9	5	2	4	7	41
(*B*) Tissues soaked for 10 min. in inoculum, then washed (slight injury)	35	21	128	30	41	147
(*C*) Tissues pricked with a needle dipped in the inoculum	282	236	247	173	214	181
(*D*) Tissues rubbed with a spatula dipped in the inoculum containing 'Celite'	114	122	199	53	134	42

The progenies were recultured a further five times and on each occasion the largest portion of the tissue was tested for its infectivity. Table 2 shows the numbers of tissue cultures from each treatment that gave a total of more than fifty lesions. In treatments *A* and *B* the number of tissues giving more than fifty lesions decreased sharply in the second or third recultures. Tissues with fewer than fifty lesions eventually gave healthy progeny. Measurements of virus content in the progenies of each inoculated tissue showed that treatments *A* and *B* only once produced more than one infected progeny. Treatments *C* and *D* were the most effective in producing complete infections in the tissues. Some progenies of such inoculated cultures were kept and have remained infected after a furthur six recultures.

Inoculation of excised root tips

Root tips about 2 cm. long were placed in a Petri dish and rubbed all over with a micro-spatula dipped in inoculum containing Celite. They were then returned to liquid medium and 2 weeks later were thoroughly washed and tested for infectivity. Extracts from most of the roots produced too many lesions to be accounted for by residual inoculum adhering to their surfaces, but the number varied and only those roots whose extracts produced many lesions when first tested remained

infected when subcultured. For example, in one experiment twelve inoculated roots gave the average numbers of lesions per leaf on *N. glutinosa* of 250, 56, 45, 33, 30, 14, 12, 10, 4, 4, 1 and 0. Although the virus had probably multiplied in at least the first five roots, only the one which gave 250 lesions remained infected after it was subcultured. White (1934) also obtained evidence of tobacco mosaic virus multiplication soon after inoculating roots, but he obtained no permanent infection. The success of our experiments can probably be attributed to the fact that the entire length of the root including the tip was rubbed with inoculum, because only the tip is used in propagation. This suggests that the virus multiplies in the inoculated cells of the root, but does not move freely as in roots of growing plants, and that the meristematic region, or at least tissues near to it, must become infected for the roots to be permanently infected. The same may be true with conditioned tissues.

TABLE 2. *The infectivity of the progenies of conditioned tissue cultures inoculated by different methods with tobacco mosaic virus*

Method of inoculation	Dates of reculturing and testing for infectivity				
	5.i.56	27.ii.56	9.iv.56	27.v.56	11.vii.56
(*A*) 0·05 ml. of inoculum poured on uninjured tissues	15/18*	10/17	5/16	5/16	5/16
(*B*) Tissues soaked for 10 min. in inoculum, then washed	10/17	6/17	6/17	6/17	6/17
(*C*) Tissues pricked with a needle dipped in the inoculum	12/13	12/13	11/12	11/12	11/12
(*D*) Tissues rubbed with a spatula dipped in the inoculum containing 'Celite'	5/11	4/10	4/10	3/6	3/6

* Denominator indicates number of tissues which survived; numerator those which produced more than fifty lesions per three half leaves of *N. glutinosa*.

This type of tissue consists of a loose mass of nodules growing independently. Each nodule has a meristematic centre surrounded by parenchyma and rudimentary vascular elements (Morel, 1948). Therefore it is likely that, only when the dividing cells are infected, is the virus necessarily perpetuated when the tissue is recultured. We show later that tobacco mosaic virus invades tissue cultures only slowly and this may explain why methods of inoculation such as pricking the tissues with needles, which place the virus in or near the dividing cells are more successful in producing infected progenies than inoculating only the superficial cells (Tables 1, 2).

Inoculation of suspensions of callus tissue cells

Table 1 shows that one of the tissues that had inoculum simply poured over it (no. 6 in treatment *A*) contained more virus than seems explicable by contamination with residual inoculum. This suggested that cells can sometimes become infected without being injured, and results in other experiments supported this conclusion. This led to the idea that dispersing tissues in liquid media might increase the chances of infection without recourse to abrasion.

Large pieces of conditioned callus with the loose growth habit were dispersed into small clumps of cells by gentle agitation for a few minutes in 20 ml. of Knop's

solution. Filtered inoculum of tobacco mosaic virus was added to the suspensions, and half the replicates were then agitated for a further 2 hr. The same number of flasks containing only culture solution and inoculum were used as controls. After 6 days the cells were removed by centrifugation, crushed with sand and resuspended in 20 ml. of sterile culture medium. The controls were also centrifuged and the infectivity of all treatments was compared in a Latin square design on *N. glutinosa*. Results in Table 3 show that most of the virus was in the supernatant fluids and little remained in the cell extracts, suggesting that the virus was released from the infected cells. Agitation after inoculation produced the most infective extracts but even without agitation, the number of lesions produced by the supernatant fluid was significantly greater than its relevant control.

TABLE 3. *The multiplication of tobacco mosaic virus in suspensions of tobacco callus tissue cells*

Treatments	Mean lesions per half leaf log $(x+1)$	Difference between supernatant fluid and control
No agitation Supernatant fluid	1·08	—
Cell extracts	0·42	—
Control	0·85	0·23*
2 hr. agitation Supernatant fluid	1·18	—
Cell extracts	0·45	—
Control	0·68	0·50*
Standard error	±0·039	±0·055

* Significant at $P=0·001$.

The results varied from experiment to experiment. Sometimes the supernatant fluids from the tissue cultures were no more infective than the controls, but usually they were more infective and sometimes produced seven times as many lesions as the controls or smaller factors that were nevertheless statistically significant. The possibility cannot be excluded that cells were injured when the tissues were dispersed and that the degree of injury varied from experiment to experiment, and this may explain the variations in virus multiplication. However, when inoculum was poured on to intact tissues growing on agar medium and sampled at different intervals, virus concentration also often increased. In one experiment lots of two tissues were sampled at 1, 6, 12 and 18 days after inoculation and the extracted sap stored at 2° C. The average numbers of lesions produced on sixteen leaves of *N. glutinosa* were 12, 18, 20 and 34, respectively, for extracts made at the four sampling times. The differences in the numbers of lesions between the samples taken at 1 and 12 days, and between 12 and 18 days after inoculation are statistically significant ($P=0·05$).

Our results suggest that undamaged cells of tissue cultures, unlike leaves, can sometimes become infected.

The spread of virus through the tissue

Once tobacco mosaic virus has entered the phloem, it moves rapidly from inoculated leaves to distant tissues. Such movement over large distances could not happen in tissue cultures where the vascular elements are rudimentary and non-functional. To study the rate of movement of virus in tissue cultures, we used the normal callus tissue which is firm and compact. The tissues were cut into strips about 15 mm. long by 4 mm. wide and inoculated either by rubbing with a micro-spatula dipped in the inoculum containing Celite or by pricking with a fine needle without Celite.

The strips to be sampled 1 day after inoculation were inoculated all over, those to be sampled at 2 days along half their length and the rest along one quarter. The pieces were inoculated in a Petri dish and carefully replaced on the medium. At intervals of 1, 2, 4, 8, 16 and 32 days, four strips inoculated by each method were sampled; each was cut into four equal parts and each part tested separately for its virus content (Table 4). In addition, three strips from the 8-day sample and two from the 32-day sample were each cut into four pieces and propagated by three serial transfers made at intervals of 3 months. At each transfer a portion of the tissue was assayed for infectivity. Table 5 gives the results of the first test only, as the healthy tissues remained healthy in the second and third tests. Tables 4 and 5 show that up to 16 days after inoculation, the virus had moved into the first uninoculated section in only two strips. In one of these recorded in Table 4, only thirty lesions were obtained suggesting that the virus had only just entered the uninoculated section. The results obtained with the 32-day sample show that the virus had moved into the first uninoculated section of all the strips and in one had reached the second section.

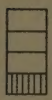
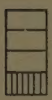
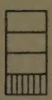
During the inoculations of the strips the limits of each inoculated portion were not marked, and it is possible that small parts of inoculated tissue might have been included in the first section said to be uninoculated. For this reason, in a further experiment similar to that recorded in Table 4, the boundaries of the inoculated sections were marked with a fine entomological needle before they were inoculated by rubbing. The results resembled those in Table 4; the average numbers of lesions per leaf of *N. glutinosa* for the 16-day sample were 94, 28, 0, 0, and for the 32-day 153, 67, 6, 1, respectively, for the inoculated and the three uninoculated sections.

Our results show, that only once did the virus enter the second uninoculated section in 32 days. This means that the virus at the most travelled about 4 mm. in this period. This estimate of the speed of cell to cell movement in tissue cultures approximates with the one made by Uppal (1934) for movement through cells of leaf parenchyma. He demonstrated that tobacco mosaic virus travelled from the upper to the lower epidermis of leaves of *N. sylvestris* Spegaz. & Comes in about 36–40 hr., and calculated the speed of movement to be about 7–9 μ/hr., which is 5–7 mm. per 32 days.

In the inoculation experiments with conditioned tissues we found that pricking with needles is a more efficient method of gaining permanent infection than is

rubbing the outer surface (Tables 1, 2), whereas in the experiments with normal tissue the two methods of inoculation produced similar results (Tables 4, 5). This difference can be explained by the difference in growth rate and internal structure of the two tissues. On average the conditioned tissue grows twice as quickly as the

TABLE 4. *The movement of tobacco mosaic virus along strips of normal tobacco tissue cultures inoculated by rubbing and pricking as shown by infectivity tests*

Days after inoculation		Average numbers of lesions per half leaf of *N. glutinosa* from four replicated tissue strips	
		Inoculation by rubbing	Inoculation by pricking
1	* 	27	19
		29	21
		32	31
		38	21
2		1	3
		4	6
		53	72
		44	61
4		0	1
		1	1
		1	7
		164	114
8		1	0
		0	0
		1	5
		84	19
16		0	0
		0	1
		30	7
		238	105
32		0	0
		1	4
		160	174
		500	375

* Schematic representation to show the section of the strip which corresponds to the lesion numbers. Shaded sections were inoculated.

normal, so that the virus will have a greater chance of becoming fully established in the normal tissue irrespective of the two methods of inoculation. Further, the normal tissue is of a compact homogeneous mass of cells, whereas the conditioned tissue is made up of loosely bound nodules which can more easily be infected by inoculation with a needle.

TABLE 5. *The movement of tobacco mosaic virus along strips of normal tobacco tissue cultures eight and thirty-two days after inoculation, as shown by infectivity tests of the progenies*

Days after inoculation		Average numbers of lesions per half leaf of *N. glutinosa*					
		Inoculation by rubbing			Inoculation by pricking		
		I	II	III	I	II	III
8	*	0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	98	0	0
		53	108	127	149	86	111
32		0	0	—	0	0	—
		0	0	—	75	0	—
		50	73	—	79	65	—
		115	147	—	84	68	—

* As in Table 4.

The role of plasmodesmata in virus movement

The view is widely held that plant viruses move from cell to cell through the protoplasmatic strands (plasmodesmata), but the only evidence for it seems to be Sheffield's (1936) claim that the guard cells of the stomata of plants infected with tobacco mosaic virus contained no inclusion bodies and that they possessed no plasmodesmata. Although Esau (1941) reported inclusions in the guard cells, this did not affect the idea that viruses move along the plasmodesmata, because she also found plasmodesmata connecting the guard cells to other cells. If plasmodesmata do provide the routes of viruses from cell to cell and are susceptible to infection, then it seemed surprising that so few infections were obtained in our experiments when tobacco mosaic virus solutions were poured over dispersed conditioned tissue cultures. With cells separated so widely, it seemed that unusually large amounts of plasmodesmatic strands should have met virus particles, yet without cells becoming infected. We therefore examined the tissue cultures for plasmodesmata by the staining techniques described by Crafts (1931). We had no difficulty in demonstrating the presence of plasmodesmata in hand-cut sections of tobacco stems and leaves, potato tubers and stems, but found none in similarly treated sections of either normal or conditioned tissue cultures.

The perforations in plant cell walls transversed by plasmodesmata can be seen after appropriate treatment, under the electron microscope. Mr H. L. Nixon has kindly compared the wall structure of our tissue cultures with that of tobacco leaves, using the method described by Thimann & Bonner (1933). This involved macerating the tissues and heating successively with 2% H_2SO_4, 2% KOH and with ethanol, and washing repeatedly with distilled water. The perforations in the cell walls of tobacco leaves are large and quite distinct (Pl. 2, fig. 2), and although the normal tissue cultures appeared to have small and not well-defined holes (Pl. 2, fig. 3) the

KASSANIS, TINSLEY AND QUAK—*Tobacco callus tissue inoculated with mosaic virus*

(*Facing p.* 18)

conditioned tissue cultures had none (Pl. 2, fig. 4). Although these results do not deny the role assigned to the plasmodesmata in the spread of viruses from cell to cell in leaves, the fact that they seem not to occur in tissue cultures, in which cell to cell movement seems as rapid as in leaves, does suggest that viruses may move from cell to cell by other means.

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EXPLANATION OF PLATE 2

Fig. 1. The three types of tobacco tissue cultures used; from left to right, normal, conditioned firm, and conditioned loose.

Fig. 2. Electron-micrograph of the cell wall of a tobacco leaf, showing two well-defined perforations, ×71,000.

Fig. 3. Electron-micrograph of the cell wall of normal tobacco tisssue culture showing what appear to be small holes, ×71,000.

Fig. 4. Electron-micrograph of the cell wall of a conditioned firm tissue apparently free from holes, ×71,000.

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